USE OF THE FLUORESCENT ANTIBODY TECHNIQUE IN THE DETECTION OF BACTERIAL BEAN BLIGHT ORGANISMS IN KANSAS

by

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INTRODUCTION

The farming of beans in Kansas and the production of certified disease-free seed in Idaho and elsewhere depend upon the early and rapid detection of disease. If this is done, then the disease may possibly be brought under control and the spread from field to field arrested. The mere presence of a trace amount of bacteria in a field can lead to disasterous results. Every bean grower, whether he be a producer of seed or of edible beans should be concerned with the early discovery of pathogenic bacteria in a crop.

The application of the fluorescent antibody technique to the detection of bacterial bean blight should aid in speeding up the process of identification of the organisms which may be causing a blight. In this way prompt and meaningful action can be taken to stop the spread of the disease.

REVIEW OF LITERATURE

Incidence

Incidence of bacterial diseases of beans have become more numerous as more land is used for growing dry beans and snap beans. In recent years, research devoted to these problems has increased considerably as the need for detection and control is realized. There is no accurate estimate as to losses incurred due to bacterial diseases, but the amount must be very great indeed. All dry bean producers surveyed in Kansas (15) said that bean diseases are a problem. Most often the diseases

are common blight (Xanthomonas phaseoli) or halo blight (Pseudomonas phaseolicola). Of these common blight appears to be more important. This is because most field beans grown have some resistance to halo blight. There are two additional bacteria which can cause disease X. phaseoli var. fuscans (fuscous blight) and P. syringae (brown spot). Damage due to fuscous blight is not known since it is often mistaken for common blight. Brown spot occurs very rarely in the United States, and is found more often in Europe. Since common and halo blight have been causing the most trouble, the major portion of literature covered concerns them. Much of what has been studied concerning one organism, such as pathology, physiology, and methods of control, often can be said to apply to the other.

Factors Influencing the Disease

Symptoms of these diseases can at times be helpful in partial diagnosis of a bean blight. However, temperature, rainfall, age of plants and other factors may alter the symptoms. Severely infected seed is shrivelled and the chances are that it will not germinate. The less severely infected seed which shows no shriveling and will germinate before showing symptoms provides the primary inoculum. Pods infected by P. syringae bend at the point of infection. Young plants are more susceptible to halo blight. As they mature, they are more likely to be infected by the common blight organism.

Temperature is an important factor in symptom production. Goss (11) and Patel and Walker (34) reported various temperature

effects on production of the halo in halo blight and production of symptoms in common blight. Common blight is generally considered a high temperature disease, whereas halo blight is considered to be a low temperature disease. Common blight occurred at low temperatures, but the incubation time was shortened to six days by keeping it at 32 C. The symptoms were less severe at lower temperatures. Halo blight, on the other hand, produced the typical halo chlorosis best at 16 C, and as the temperature rose, the halo became less apparent and disappeared at 28 C. At the higher temperatures there was an increase in the number of infections per leaf along with the modification of the halo. Temperature is not as important a factor in the time needed for symptom development in halo blight as it is in common blight.

Humidity is a factor which tends to effect common blight development, but not halo blight. Symptoms of common blight are more severe in a low humidity environment. Halo blight symptoms appear equally severe in high humidity as well as in low humidity.

Plant nutrition can also play a part in production of symptoms. Plants supplied with Hoagland's basal nutrient solution showed maximum vegetative growth as well as greatest common and halo blight development (33). Increased amounts of this balanced solution suppressed disease development. Also, very low and very high levels of nitrogen, potassium, and phosphorus retarded the severity of both halo and common blights. If only high amounts of potassium and phosphorus were added, common blight development was enhanced.

Phytopathogenic pseudomonads are gram negative rods with one to six polar flagella. Their optimum growth temperature is 27-30C. They will remain viable on beef-extract peptone agar for months, but will not do so on potato dextrose agar. Kelman and Pearson (22) stated that cultures of P. solanacearium can be kept in sterile water at 22 C for 18-24 months without losing their virulence. The production of a halo around the lesion in halo blight is due to an exotoxin produced by P. phaseolicola. This toxin, however, is not produced at temperatures above 20 C in pure culture according to Zaumeyer and Thomas (52).

Description of Organisms

The genus Xanthomonas is characterized by short gram negative rods with one polar flagellum. The colony growth is slimy on sugar-containing media, and yellow due to water insoluble carotenoid pigment. Xanthomonads will remain viable on potato dextrose agar and will die on sugar-free beef peptone or yeast extract agar, in contrast to pseudomonads. Xanthomonads were found to grow on a minimal media of NH_4Cl, glucose and salts. X. phaseoli var. fuscans differs from X. phaseoli in that it produces a red pigment in media containing tyrosine which soon turns brown and diffuses throughout the media. There is no correlation between the amount of pigment produced and virulence (49). Another extracellular produce formed by xanthomonads grown on a carbohydrate-rich medium is a heteropolysaccharide material of high molecular weight although no increase is

noted in the capsule size (8). This polysaccharide material which, if not removed, gives a cross reaction between species. If cells are washed in warm saline, this gum is eliminated and cultures react more specifically upon agglutination testing. Xanthomonads do not tolerate high salt concentrations. Two to three per cent NaCl often retards growth and all of them fail to grow in medium containing 5% NaCl.

Pathology and Histology

Various biochemical studies have been done regarding the interaction of host and parasite, or on the parasite alone. In the case of halo blight it was found (35) that the invading organism caused an increase in ornithine, histidine, methionine, asparagine, glutamine, B-alamine and lysine in inoculated leaves. In uninoculated leaves on the same plant that became chlorotic the amount of these compounds was even greater. In another study (41) using the same host and parasite (Phaseolus vulgaris and P. phaseolicola, respectively) changes in the protein pattern of infected leaves on the chromatograph were noted. Changes were different for susceptible and resistant varieties of beans. New enzymes that were detected were: dehydrogenases, oxidases (peroxidases), acid phosphotase, and esterase. These compounds appeared to be of bacterial origin. A catalase band of bacterial origin was found after one day. Virulent bacteria gave higher catalase activity than non-virulent bacteria. Within two days peroxidase activity of susceptible bean leaves

decreased or remained the same whereas in resistant varieties it increased. Rudolph suggests (41) that the host resistant hypersensitive reaction is related to host peroxidases, which are suppressed by bacterial catalase in the susceptible combinations. Deverall and Walker (7) noted that another difference in resistant and susceptible varieties of P. vulgaris to P. phaseolicola is the rate of oxygen uptake. Hydroxylamine, amide, and cyanide inhibited oxygen uptake 20, 30, and 40%, respectively.

Information as to how bacterial bean diseases are spread often appears conflicting. Two facts seem to be agreed upon by most: 1) bacterial bean diseases often are seed borne, and 2) it does not require many infected seeds per acre to cause a serious epiphytotic. Walker and Patel (47) found that as few as twelve infected seeds per acre is sufficient to do serious damage.

Zaumeyer and Thomas (52) stated that the soil is an unlikely source of carry over of bacteria. Experiments by Hedges (19) show that beans infected with halo blight that were plowed under in the fall did not contaminate a crop of healthy beans planted the following spring. An agricultural experiment station bulletin from Kansas State University (15) states that blight bacteria can overwinter two to three years on bean seed or plant material. Fenwick and Dean (9) state that halo blight bacteria can overwinter in or on infested seed and plant debris. In Colorado three- to four-year rotations are practiced and soil borne inoculum is not believed to exist (31).

Splash dispersal of organisms in a field is also an area for some question. Walker and Patel (47) report that halo blight spread from inoculated areas of a bean crop was due to splash dispersal and directed by prevailing wind. Dispersal was up to 85 ft. from the original inoculum. In a similar experiment Zaumeyer and Thomas (52) report only slight evidence very late in the growing season that indicated any spread of common blight by an overhead irrigation system. Others (9, 15) feel that rain, especially wind-driven, will aid in dispersal of the disease.

Upon examination of the pathology and histology of host and parasite, it is noted that infection of the seed takes place via the funiculus or micropyle (51). The bacteria remain just under the seed coat. Upon germination, if the bacteria are at the distal part of the cotyledon, they may not traverse the tissue fast enough to enter the stem before the abscission layer forms. If this is the case, the plant will remain uninfected. The most damage seems to occur when the optimum growth of the host is going on (52). The action of the bacteria infecting beans is by extracellular enzymes which dissolve the cell walls.

Methods of Detecting Organisms

The host range of the different bean-infesting organisms varies according to the particular organism. After testing a number of bean varieties, Walker and Patel found that one of their cultures of P. phaseolicola would infect all beans tested, and the other cultures

would infect everything except Red Mexican UI-3. On this basis, the latter strains were designated as Race 1 and the former strain as Race 2 (48). Guthrie has noted (18) by means of gel diffusion technique that there is no serological difference between Race 1 and Race 2. This statement appears to be in opposition to what he previously said concerning the production of antisera specific for Race 1 and Race 2 of P. phaseolicola. Pseudomonas phaseolicola, beside being able to go to a rather large variety of beans, was also able to multiply on roots of wheat, tomato, and soybean in the laboratory (52). Field tests, however, showed that the organism could not be recovered from the roots of inoculated wheat.

Xanthomonas phaseoli and X. phaseoli var. fuscans can be differentiated in that X. phaseoli has a wider host range. It can go to Phaseolus spp., Dolichos spp., and Lupinus spp., whereas X. phaseoli var. fuscans can go only to Phaseolus spp.

Various methods have been used to obtain an accurate and rapid diagnosis of bacterial diseases on plants. Kelman and Pearson (22) have used a technique with tetrazolium chloride in the media which enables them to differentiate between pathogenic and non-pathogenic strains of the same culture by clone appearance. Pathogenic clones of P. solanacearum appear white on this media, and the non-pathogenic ones appear red or pink. Pitts and Pierce (38) devised a method for testing pathogenicity of organisms by stabbing bean pods and observing the development of water-soaked lesions that form after four days.

For isolation of the bacteria from diseased leaves, another puncture method is used (12) in which the inoculating needle is pushed through the leaf and directly into the agar. A streak isolation is then made from there.

Buddenhagen, Sequeira, and Kelman (2) have used a host range to differentiate races of bacteria. Symptom expression under field conditions on a series of differential hosts is more meaningful than results based on routine biochemical reactions or phage testing, according to the authors.

Perhaps the most rapid and accurate method for detection of bacteria is by serological means. In the past few years the number of papers on use of this technique has been increasing steadily. Fluorescent antibody technique, tube and micro-agglutination tests, bentonite flocculation test, micro-percipitin tests, and formamide tests are some of the methods employed.

Guthrie (18) found serum prepared against X. phaseoli, X. phaseoli var. fuscans, Corynebacterium flaccumfaciens, as well as Race 1 and 2 of P. phaseolicola to be highly specific to his original culture. Morton (26, 27, 29, 30) found that serological methods gave accurate results when used on plant extracts. It was found (37) that one cannot distinguish between virulent and avirulent strains by serological means. Pathogenicity evidently is not associated with a type of antigenic structure.

Elrod and Braun (8) did an extensive study on serological cross reactions of the genus Xanthomonas and their relationship to one another.

They broke the genus into five major groups based on serological closeness: the a. Vascularum group, b. Phaseoli group, c. Campestris group, d. Pruni group and e. Translucens group. Wernham (50) criticized this grouping system saying that the use of host range is better than serology for Xanthomonas identification. He argued that the host infection range did not correlate with the serological cross reactions. Corroborating this, Schnathorst (42) stated the same idea, using the example of X. phaseoli and X. malvacearum being serologically close, but only one of these organisms is pathogenic to bean.

Other methods have been used to detect bean blight organisms such as phage typing and using formamide. Wallen and Sutton (49) and Quinon, Aragaki and Ishii (40) both have reported using phage for identification. Xanthomonas phaseoli could be differentiated from X. phaseoli var. fuscans, and strains of P. solanacearum could be identified in the same manner. The formamide method (36) of identification used antisera prepared against whole bacterial cells and the extracted polysaccharide material from the cells as antigen. This method does not differentiate between strains of P. solanacearum, the organism used.

Control of bacterial diseases is rather difficult since detection of small amounts of diseased seed is hard to accomplish, soaking seeds in various antibacterial agents does not penetrate to the inner side of the seed coat to kill, and stopping the disease once symptoms show is very difficult.

Various safety precautions such as crop rotation, using certified seed, avoiding overhead irrigation, and not working fields after a

rain may help in decreasing the incidence and severity of bean blights. Seed treatment, especially with antibiotics, has been claimed by some (46, 52) to have an effect in killing the bacteria. Others (39, 43) feel that there is little to be gained by their use. Perhaps the most desirable and long-lasting method of eliminating the bean blight problem is by developing bean plants that are resistant or tolerant to the phytopathogenic bacteria.

METHODS AND MATERIALS

Organisms

For this study five organisms were chosen. These were X. phaseoli var. fuscans (obtained from A. Eisenstark), X. alfalfae (obtained from D. Stuteville), P. syringae, strain P-134 (obtained from D. Hildebrand), and P. phaseolicola, Races 1 and 2 (obtained from J. Guthrie). All of these organisms were checked for purity by four flame streak isolation and Gram staining. Clones appeared uniform, and staining revealed Gram negative non-sporeforming rods of uniform size.

Preparation of Antigen

The organisms were grown on potato dextrose agar (PDA) consisting of potato, 2% dextrose, and 1.7% agar. Inoculation of the media was done by pouring sterile distilled water on a 48-hour-old slant of the organism, loosening them with a sterile inoculation loop, and spreading 1 ml of the suspension onto PDA in a Blake bottle. After

48 hours of incubation at 26C, the organisms were washed off the PDA with sterile normal saline and Gram stained to assure maintenance of pure cultures. The bacteria were then centrifuged, resuspended in the normal saline and the process repeated twice more. After the last centrifugation, the bacteria were suspended in 0.3% formalized saline and sonicated to break up the cells. A Bronson S-75 sonicator was used at 4 amperes for 6 minutes. A simple methlyene blue stain was run on the sonicated cells to assure that lysis had been complete. The lysed cells were then diluted with more formalized saline until a reading of 20% transmittance of 500 mu was reached on a Bosch and Lomb Spectronic 20. This antigen was then stored in a refrigerator at 5C until used.

Preparation of Antisera

Rabbits were bled previous to inoculations to see if there had been any previous exposure to the antigens used. This was done by tube agglutination testing. No evidence of exposure to the antigens was apparent. Following this, the antigens were injected into the rabbits intravenously (Table 1).

After a 7-day rest period following the last injection, a small amount of serum was obtained by ear bleeding in order to determine the titer. The tube agglutination technique was used to determine the titer. If the titer proved satisfactory, the rabbits were bled by cardiac puncture, the blood centrifuged to obtain the serum, and the serum frozen until used.

Table 1. Immunization schedule.

Antigen	Schedule for each antigen	
	Day	Amount (ml)
X. phaseoli	1	0.5
X. phaseoli var. fuscans	4	1.0
X. alfalfae	7	1.5
P. phaseolicola (Race 1)	10	2.0
P. phaseolicola (Race 2)	13	2.0
P. syringae (P-134)	16	2.0
	19	2.0
	26	bled

Fluorescent Labeling

Fluorescein isothiocyanate (FITC) on celite (10%) (Calbiochem) was the dye used to conjugate to the serum a gamma globulin fraction. The method used consisted of shaking for 3 minutes a mixture of 3 ml of rabbit antiserum, 3 ml of 0.05 M sodium carbonate-bicarbonate buffer of pH 8.5 and 10-40 mgm. of the dye. The mixture was then centrifuged for 10 minutes at 2,000 rpm in a Sorvall SS-3 centrifuge to remove the celite. Following this, the supernatant was put through a column (2.8 x 10 cm) of Sephadex G- 25 (Pharmacia Uppsala, Sweden). Separation of the labeled protein from the unreacted dye was accomplished in this manner, and the labeled serum assumes a pH of 6.5 which is the pH of the phosphate buffer used

to develop the Sephadex column. The labeled serum was collected in approximately 9 ml of effluent which is an increase of 50-100%. This process requires ten to fifteen minutes. The column was then emptied, the Sephadex washed in a beaker until clean, and repacked in the column.

Fractionation of Serum Globulins

In the preparation of the gamma-globulin fractions of these antisera, equal volumes of saturated ammonium sulfate, kept at room temperature, and the undiluted serum which had been kept in an ice bath were mixed. The precipitated globulin was allowed to stand overnight at 0-5C. It was then centrifuged in a Sorval SS-3 at 5,000 rpm for twenty minutes in order to separate the sediment. The supernatant was then discarded and the crude globulin was dissolved by slowly adding distilled water. When the suspension had become homogenous, an amount of saturated ammonium sulfate equal to the volume of water was used to dissolve the precipitate. The precipitated globulin was centrifuged immediately, the supernatant discarded, and the precipitate resuspended. This process was repeated three times or until most of the hemoglubin was eliminated. The last resuspension of the globulin precipitate was done in as little water as possible, and then it was dialyzed against 0.85% sodium chloride at 5C until no more sulfate could be detected in the liquid outside the dialysis sac. Detection of sulfate was done using saturated barium chloride and an equal volume of water from outside the sac; a clouding of this mixture indicated sulfate was still present.

Absorption of Common Antibodies

A 2 ml quantity of each of the antigens used in the immunization procedure previously described was added to 1.5 ml of sera, with the exception of the antigen for which the serum was made. These mixtures were agitated for thirty minutes at room temperature and then centrifuged at 2,000 rpm for one hour. The process was then repeated. One percent of the heterologous gamma globulin (unlabeled) was added to the absorbed labeled globulin. The preparations were kept frozen until ready to be used.

Storage of Organisms

The five organisms being used in this experiment (X. phaseoli var. fuscans, X. alfalfae, P. syringae, and P. phaseolicola (Race 1 and 2) were each placed in a refrigerator. After one year four loops of water from each tube were streaked on PDA and incubated at 26C to determine the survival ability of the organisms.

Bean Pod Inoculation Technique

The technique of Pitts and Pierce (38) was used in order to determine bacterial pathogenicity. It consisted of inoculating bean pods with a sharp instrument such as a probe having the organism on the tip. The pod then was placed in a screw cap test tube which contained approximately 0.5 inches of sterile water and stored at 26C. If the organism was pathogenic, water-soaked lesions would appear on the pods. This method was employed in checking the

pathogenicity of the five organisms used in this study as well as other unknowns.

Isolation of Unknown Organisms

The method used was that of Goth (12). A leaf containing a lesion was held over a plate of PDA and a sterile inoculating needle was pushed through the lesion and directly into the agar. A sterile glass rod was then used to spread the bacteria over the surface of the PDA. This was then incubated at 26C for 24-48 hours. When necessary, four, flame streak isolations were done to insure pure cultures. The pure cultures were grown on PDA slants and stored at 0-2C until used.

For isolation of organisms from seed, a 1% Na $HclO_3$ solution was used to soak the seeds for 5 minutes. The seeds were then washed in sterile water and placed in a test tube with 0.25 inches of sterile water and allowed to germinate in an incubator at 26C. When the water appeared turbid after 3-4 days, a loopful of the water was streaked on PDA and isolated clones were transferred to slants.

Direct Method of Staining

The organism being tested was dispersed in a small drop of water onto a slide, allowed to air dry, and was heat fixed by passing through a bunsen burner flame three times. A drop of labeled antiserum was placed on the slide and incubated in a water-saturated atmosphere at 37C for 30 minutes. The slide was then washed for

three successive ten-minute rinses in phosphate buffered saline (PBS). The slide was mounted with a drop of PBS containing 20% glycerol, covered with a cover slip and sealed with lacquer. The preparation was refrigerated at 0-4C for one-half hour or more and then examined under a Leitz Ortholux microscope with ultra violet light.

Indirect Method of Staining

Organisms were fixed in the same manner as described for the direct method. A drop of unlabeled antiserum was applied and let stand for thirty minutes in a water-saturated atmosphere at 37C. The slide was washed for three successive ten-minute rinses in PBS, drained, but not dried. A drop of labeled sheep anti-rabbit serum was then added and allowed to stand for thirty minutes in a water-saturated atmosphere at 37C. It was then washed three times in PBS, shaken dry, covered with 20% glycerol in PBS, sealed, and refrigerated. After about one-half hour it was examined under the ultra violet light microscope as was done when the direct method was used.

RESULTS

The organisms tested were those which were used as antigens plus some which were isolated from various bean crops in western Kansas in 1966. In each case, a control puncture was made with a

sterile probe at the top of each pod. A visible exudate was considered definite indication, and a water-soaked appearance without exudate was considered slight infection.

Table 2. Pathogenicity as determined by bean pod inoculation.

Organism	Appearance
X. phaseoli	Definite infection
X. phaseoli var. fuscans	Slight infection
X. alfalfae	Slight infection
P. phaseolicola (Race 1)	Slight infection
P. phaseolicola (Race 2)	Slight infection
P. syringae	Slight infection
Leoti isolate	Definite infection
Ulysses isolate	Definite infection
Garden City isolate A	Slight infection
" " " B	Slight infection
" " " C	Definite infection
" " " D	Definite infection
" " " E	Definite infection

Pseudomonads in general grow in a more varied set of conditions than any other type of organism. Their ability to withstand such adverse conditions as no nutrients and low temperatures for extended periods of time can be noted by the fact that these were the only

Table 3. Pathogenicity and survival of organisms stored on PDA for 1 year.

Organism	Growth on PDA	Pathogenicity
X. phaseoli	growth	slight
X. phaseoli var. fuscans	growth	slight
X. alfalfae	growth	slight
P. phaseolicola (Race 1)	none	none
P. phaseolicola (Race 2)	growth	none
P. syringae	growth	slight
Leoti isolate	growth	slight
Ulysses isolate	growth	none
Garden City isolate E	growth	definite

Table 4. Survival of known organisms in distilled water for 1 year at 5C.

Organism	Growth on PDA
X. phaseoli	none
X. phaseoli var. fuscans	none
X. alfalfae	none
P. phaseolicola (Race 1)	none
P. phaseolicola (Race 2)	growth
P. syringae	growth

type of organisms to survive in cold distilled water for one year. However, the possibility of both xanthomonads and pseudomonads overwintering on plant debris in the soil cannot be ruled out on the basis of the above data. A general decline in pathogenicity can be seen in some instances.

Table 5. Titers as determined by the tube agglutination test.

Antigen	Unlabeled, Unabsorbed sera	Unlabeled, Absorbed sera	FITC Labeled Gamma globulin
X. alfalfae	1:1280-1:2560	1:80-1:160	1:80-1:160
X. phaseoli var. fuscans	1:1280-1:2560	1:80-1:160	1:640-1:1280
X. phaseoli	1:20-1:40	0	0
P. syringae	1:1280-1:2560	1:320-1:640	1:640-1:1280
P. phaseolicola (Race 1)	1:160-1:320	0	1:80-1:160 (unabsorbed)
P. phaseolicola (Race 2)	1:40-1:80	0	1:10-1:20 (unabsorbed)

type of organisms to survive in cold distilled water for one year. However, the possibility of both xanthomonads and pseudomonads overwintering on plant debris in the soil cannot be ruled out on the basis of the above data. A general decline in pathogenicity can be seen in some instances.

From the above data it can be noted that there is very little, if any, difference in the antigens present in Race 1 and Race 2 of

P. phaseolicola. In addition, the titer of X. phaseoli was not deemed sufficiently high to be of use. Xanthomonas phaseoli var. fuscans antiserum was used for the detection of X. phaseoli.

The method which assured no cross reaction and, therefore, eliminated non-specific fluorescence was the addition of a small amount of unlabeled gamma globulin of the cross reacting species. Absorption alone was not sufficient. In addition, repeated absorption lowered the titer of the gamma globulin which decreased the degree of fluorescence.

Table 6. Identification of known organisms using fluorescence (direct method).

Labeled gamma globulin	Unlabeled heterologous gamma globulin	Antigen	Fluorescence
X. phaseoli var. fuscans	none	X. phaseoli	+4
X. phaseoli var. fuscans	none	X. alfalfae	+2
X. phaseoli var. fuscans	X. alfalfae	X. phaseoli	+4
X. phaseoli var. fuscans	X. alfalfae	X. alfalfae	none
X. alfalfae	none	X. alfalfae	+4
X. alfalfae	none	X. phaseoli	+1
X. alfalfae	X. phaseoli var. fuscans	X. alfalfae	+4
X. alfalfae	X. phaseoli	X. phaseoli	none
P. syringae	none	P. syringae	+4
P. syringae	none	P. phaseolicola (Race 2)	+3

Table 6 (concluded).

Labeled gamma globulin	Unlabeled heterologous gamma globulin	Antigen	Fluorescence
P. syringae	P. phaseolicola (Race 2)	P. syringae	+4
P. syringae	P. phaseolicola (Race 2)	P. phaseolicola (Race 2)	+1
P. phaseolicola	none	P. phaseolicola (Race 2)	+3
P. phaseolicola	none	P. syringae	+2
P. phaseolicola (Race 2)	P. syringae	P. phaseolicola (Race 2)	+3
P. phaseolicola (Race 2)	P. syringae	P. syringae	none

+4 = highest fluorescence
+3 = bright fluorescence
+2 = moderate fluorescence
+1 = weak fluorescence

Table 7. Identification of unknown organisms using labeled absorbed gamma globulin with unlabeled heterologous gamma globulin.

Source of bacteria and year isolated	Clone color	Gamma globulin used			
		X. phaseoli var. *fuscans*	*X. alfalfae*	*P. syringae*	*P. phaseolicola*
Garden City (1966)	yellow	+1	--	--	--
Ulysses (1966)	yellow	+1	--	--	--
Leoti (1966)	yellow	+1	--	--	--
Safeway Pod (1967)	yellow	--	--	--	--
Safeway Pod (1967)	yellow	+3	--	--	--
Garden City (1967)	yellow	+4	--	--	--
Garden City (1967)	yellow	--	--	--	--
Garden City (1967)	white	--	--	--	+3
Garden City (1967)	white	--	--	--	+3
Controls used:					
X. phaseoli	yellow	+4	--	--	--
X. alfalfae	yellow	--	+4	--	--
P. phaseolicola (Race 2)	white	--	--	--	+3
P. syringae	white	--	--	+3	--

+4 = highest fluorescence
+3 = bright fluorescence

DISCUSSION

The results of the bean pod inoculation test for pathogenicity showed that the known organisms that were used were only slightly pathogenic. This is not uncommon among the organisms kept on laboratory media for any period of time. However, since pathogenic strains of the bacteria used are serologically indistinguishable from non-pathogenic strains, this factor does not appear to be critical. Storage for one year on PDA showed a slight decline in pathogenicity. Presumably after a longer period of storage, pathogenicity could be totally lost. The possibility of overwintering on plant material cannot be discounted. Judging by the results of the survival of organisms in distilled water, the pseudomonads appear to be able to stand more stringent conditions than can the xanthomonads.

However, since there are conflicting findings already reported, more research is needed before definite conclusions can be drawn. Zaumeyer and Thomas (52) and Hedges (19) found no evidence of overwintering; yet Greig and Gevin (15) along with Fenwick and Dean (9) found that overwintering is possible.

In preparing antisera for labeling, a high titer was necessary since dilution during absorption and labeling would occur. Xanthomonas phaseoli did not give a satisfactory titer, and P. phaseolicola gave a functional but low titer. Using the gamma globulin fraction of the various sera did increase the titer so that after labeling, a functional titer remained. Since X. phaseoli and X. phaseoli var. fuscans

were not absorbed against each other, I felt it safe to use the latter antiserum to detect X. phaseoli. In a subsequent report by Guthrie to the Phytopathological Conference in Washington, D. C. (1966), he stated that Race 1 and Race 2 of P. phaseolicola are not distinguishable by serological means. Therefore the use of only one of the P. phaseolicola sera was deemed necessary.

The titer achieved proved to be in direct proportion to the amount of fluorescence produced. The best combination found was by using labeled absorbed gamma globulin combined with heterologous gamma globulins. In this way any possible cross reaction sites on the antigen were combined more rapidly with the unlabeled gamma glubulin thus blocking the labeled gamma globulin. This eliminated the possibility of non-specific fluorescence.

Once satisfied that the system could be relied upon, the organisms isolated from field samples were tested. A preliminary selection could be made as to which clones should be tested by noting the color. Xanthomonads are characterized by a bright yellow color and pseudomonads by a white color. Since some of the organisms tested had been isolated the previous year and stored on PDA, their degree of fluorescence was weaker. The more recent isolations were more defined.

SUMMARY

Five organisms known to infect beans were selected. These were cultivated and inoculated into rabbits. The antisera obtained was then labeled with fluorescine isothiocyanate and used to identify

bacteria under an ultra violet microscope. Absorption of common antigens assured no non-specific fluorescence.

Survival of the organisms for one year on PDA, and in distilled water was tested. Growth on PDA was observed, but only growth of pseudomonads after storage in water was observed.

A bean pod inoculation test was run to test pathogenicity of the organisms. It was found that keeping the bacteria for one year decreased their infectivity.

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USE OF THE FLUORESCENT ANTIBODY TECHNIQUE IN THE DETECTION OF BACTERIAL BEAN BLIGHT ORGANISMS IN KANSAS

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Fluorescent antibody technique was applied to the detection of bacteria which infect Phaseolus spp. Five organisms were used as antigens: Xanthomonas phaseoli, Xanthomonas phaseoli var. fuscans, Xanthomonas alfalfae, Pseudomonas syringae, Pseudomonas phaseolicola. These were injected intravenously into rabbits. Antiserum was then obtained by cardiac puncture, and the blood centrifuged. Common antigens were then absorbed out, and the sera were labeled with fluorescein isothiocyanate. Tests using known cultures proved reliable, and the unknown organisms were then tested. Of the samples tested, X. phaseoli was most often found.

Survival of these organisms for 1 year on potato dextrose agar (PDA) and in distilled water was tested. Survival on PDA by all organisms was observed. The pseudomonads, however, were the only ones capable of storage in water for 1 year. The possibility of these organisms overwintering on plant debris in the soil is still open to debate.

The organisms were also tested for pathogenicity by inoculation into bean pods. A water-soaked zone indicated pathogenicity. There is, however, no antigenic difference between pathogenic and non-pathogenic strains of the same organisms.